

**REPRODUCTION AND GENETIC PARENTAGE IN A PAIR-LIVING
HERMAPHRODITE, THE INTERTIDAL LIMPET SIPHONARIA GIGAS**

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Abstract

Pair-living is a common social system found across animal taxa, and the relationship between pair-living and reproduction varies greatly among species. *Siphonaria gigas*, a hermaphroditic pulmonate gastropod, often live in pairs in the rocky intertidal. Combining genetic parentage analysis using four polymorphic microsatellite loci with behavioral observations from a 10-week field study, I provide the first description of the mating system of a *Siphonaria* species incorporating genetic data. *S. gigas* mated both within-pair and extra-pair and three out of four paired *S. gigas* individuals produced egg masses with extra-pair paternity. Multiple paternity was detected, but at a relatively low frequency (19% of egg masses) compared to other marine gastropods. Behavioral data indicate one potential advantage of pair-living: paired *S. gigas* produced almost twice as many egg masses as their solitary counterparts over four reproductive cycles. These observations, together with constraints on the movement of *S. gigas*, suggest that pairing may be a strategy to ensure mate access.

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Introduction

Animal mating and social systems both influence and are influenced by the spatial distribution of individuals within populations. As sexual reproduction involves the union of gametes from different individuals, spatial proximity is often necessary for animals to mate and reproduce. Pair-living or social monogamy is both a spatial phenomenon and social system in which pairs of conspecifics live together for an extended period of time (Wickler and Seibt 1981). Pair-living is found across a variety of organisms with different modes of reproduction, from snails to primates, and including hermaphrodites and animals with separate sexes (crustaceans: Detto and Backwell 2009, Baeza et al. 2009; polychaetes: Sella and Lorenzi 2000; reef fish: Brandl and Bellwood 2014; mammals: Lukas and Clutton-Brock 2013; birds: Griffith et al. 2002, Black 1996).

Extensive studies in vertebrates have generated several hypotheses for the evolution of pair-living, which is thought to result from natural selection favoring behaviors such as biparental care, cooperative territoriality, and mate guarding (Emlen and Oring 1977, Lukas and Clutton-Brock 2013). However, not all pair-living species have these characteristics (e.g., Baeza et al. 2016, Sella and Lorenzi 2000, Wong and Michiels 2011), and less is known about the evolutionary consequences and potential drivers of pair-living in these species. Baeza and Thiel (2007) proposed a conceptual model to predict the mating systems of symbiotic crustaceans based on ecological traits. Under this model, monogamy is the optimal mating system when hosts are rare and support few individuals and the cost of switching hosts is high (Baeza and Thiel 2007). More broadly stated, pair-living may be favored when refuges are scarce and moving between them is risky (e.g., due to predation), constraining an individual's ability to find

a mate. This idea has been supported in studies of symbiotic marine invertebrates (Baeza 2008, Baeza 2010, Pfaller et al. 2014), however, it is unknown whether environmental constraints might favor social monogamy in free-living organisms. Biparental care, cooperative territoriality, mate-guarding, and Baeza and Thiel's environmental constraints hypothesis share the assumption that pair-living confers adaptive benefits in terms of survival, reproduction, or both. Thus, testing hypotheses for the evolution of pair-living in any organism relies on knowledge of its ecology, reproductive biology, and mating system.

Pair-living animals may or may not mate with one another, whereas sexual monogamy entails exclusive reproduction between two individuals. The degree of sexual monogamy varies widely among socially monogamous species, and genetic studies of pair-living animals have revealed high frequencies of mixed-paternity (e.g., Ophir et al. 2008, Griffith et al. 2002) or mixed-maternity broods (e.g., DeWoody et al. 2016) as well as sexual monogamy (e.g., Piper et al. 1997). Multiple paternity occurs when a female mates with and produces offspring sired by multiple males; this pattern has been documented in many animal taxa and is thought to confer adaptive benefits, for example, by increasing the genetic diversity of a female's offspring (Arnqvist and Nilsson 2000, Griffith et al. 2002).

To my knowledge, only one study has described pair-living in a mollusk, *Siphonaria gigas*, an intertidal gastropod, which lives in pairs at a rocky intertidal site on the Pacific coast of Panama (Lombardo et al. 2013). *Siphonaria* is a genus of hermaphroditic pulmonate gastropods with internal fertilization, sometimes referred to as "false limpets" (Hodgson 1999), and includes over 40 species found on intertidal shores world-wide (Dayrat et al. 2014). *S. gigas*, the largest

members of *Siphonaria*, are distributed from Mexico to Peru in the tropical Eastern Pacific Ocean (Keen 1971). Individual *S. gigas* establish “home scars” by growing their shells to precisely fit the substrate at a fixed location, leaving their home scars for limited periods to graze on encrusting algae and cyanobacteria and reproduce (Hodgson 1999, Levings and Garrity 1984).

The movement of *S. gigas* is highly constrained spatially and temporally by environmental conditions. Limpets leave their home scars only on falling and rising tides while the substrate is wet and while being splashed by waves; they are not active when immersed in water and are rarely seen moving over dry rocks (Garrity 1984). Homing is critical for survival, as limpets face greater risk of mortality due to desiccation and predation when off scar (Garrity and Levings 1983). When they do leave their scars, individuals typically move within a meter radius and remain active for an hour or less (Levings and Garrity 1984, J. Schaefer & J. H. Christy personal observations). *S. gigas* do not self-fertilize, so access to mates is necessary for reproduction, yet restricted movement suggests their opportunities to encounter and mate with other individuals may be limited (Lombardo et al. 2013).

Lombardo et al. (2013) surveyed a population of *S. gigas* at Punta Culebra, Panama and found that 75% of limpets occurred in pairs on adjacent home scars, typically so close that their shells touched when both were on their scars (Lombardo et al. 2013, Levings and Garrity 1986). Pairs of *S. gigas* can persist for months (Levings and Garrity 1986) and some pairs have persisted at least five years (J. H. Christy personal observation). While the majority live in pairs, the remaining, unpaired limpets are found as solitary individuals or in groups of more than two. Thus, intrapopulation variation in social status provides an opportunity to measure the

reproductive consequences of pair-living in a natural setting. *S. gigas* lack parental care, mate-guarding, and territorial behavior, so alternative ecological factors must lead to pair-living.

In 2016 I revisited this same population at Punta Culebra to gather behavioral, reproductive, and genetic data for the present study. The aim of this study was to describe the mating system of *S. gigas* in the context of pair-living and determine the relationship between the spatial distribution and reproduction of *S. gigas*. To achieve this, I combined observations of limpet behavior and reproductive output with genetic parentage analysis of the same limpets and their putative offspring. Specifically, I tested whether pairs of limpets are sexually monogamous and whether multiple paternity occurred within egg clutches. This paper provides the first description of the mating system of a *Siphonaria* sp. utilizing genetic parentage analysis.

Methods

This study combined field observations of *Siphonaria gigas* behavior and egg mass production with genetic analyses of adults and their putative offspring. Behavioral and reproductive data were collected in Summer 2016 and analyzed in Fall 2016–Spring 2019, and the genetic data were collected and analyzed from Fall 2016–Spring 2019.

Study organism and sampling location

The field portion of this study was conducted at Punta Culebra, Panama (8° 54' N to 79° 31' W), in an intertidal area of large granite boulders. A population *S. gigas* at Punta Culebra was previously surveyed, revealing that individuals preferentially live along horizontal fissures in the rock, with some on exposed horizontal or vertical rock faces and very few inhabiting tidepools (Lombardo et al. 2013). Each limpet has a home scar, which is recognizable by its lighter coloration and the absence of the algal crust that covers most of the substrate. *S. gigas* exhibit strong reproductive synchrony and produce benthic egg masses on the semimonthly neap tides, and each egg mass contains more than 75,000 embryos on average (Levings and Garrity 1986). Embryos develop in the egg masses for 7–10 days before hatching and entering a planktonic stage.

The “social status” of *S. gigas* individuals can be classified as paired, solitary, or grouped based on their home scar location. Paired limpets have home scars immediately adjacent to one another; grouped limpets are those living in clusters of three or more; solitary limpets are not directly adjacent to any other limpets when on their scar (Fig. 1). Lombardo et al. (2013) showed that pairing occurs significantly more often than expected by chance, with 75% of *S. gigas* in

rock fissures at Punta Culebra living in pairs. The frequency of pairing was also negatively related to density, indicating pair formation does not result from crowding (Lombardo et al. 2013).

In May 2016, I tagged 37 solitary individuals and 74 paired individuals (37 pairs) of *S. gigas* by adhering spots of waterproof epoxy putty (PC Marine) to their shells and writing unique identification numbers on the putty spots. The shell length of each limpet was measured to the nearest mm along the anterior-posterior axis, and the distance between solitary limpets and their closest neighbor was measured to the nearest 0.5 cm. Shell lengths of the paired and solitary limpets that were marked were compared using a Wilcoxon rank-sum test.

Behavioral observations and egg mass production

I visited the study site daily from May 13–July 21, 2016 and monitored the movements, social status, mating behavior, and reproductive output of marked limpets. Observation periods were during daytime low tides and lasted for 1–4 hours. Due to the large size of the study site, I was not able to observe all marked limpets at all times, so mating and behavioral observations were not comprehensive. I recorded any changes in social status and home scar location. When marked limpets mated, indicated by two limpets positioning themselves head-to-head with their shells raised and genital pores in contact (Hodgson 1999; Fig. 1b), I recorded the date and identities of the mating pair. *S. gigas* are inactive while submerged in water (Garrity 1984), so it is unlikely that limpets mated during high tide. However, they may have mated during nighttime low tides.

I recorded the number of egg masses produced by each marked limpet over four reproductive cycles. Egg mass production occurred four times over the study period: May 28, June 13, June 28, and July 12 (± 1 day). On egg production days, I arrived at the site just as the tide was falling and remained there until it rose again (~ 5 hours) in order to observe as many egg masses being deposited as possible. I noted which individuals produced egg masses and marked the location of each egg mass with an epoxy tag on the rock for later sampling (see *Tissue collection for genetic analysis*). To allow the embryos to grow larger, egg masses were sampled six days after deposition; to protect the masses from fish predation prior to sampling, I installed predator exclosures over each egg mass by affixing wire mesh caps to the rock with waterproof epoxy putty.

To compare total egg mass production between paired and solitary limpets, I constrained the dataset to marked limpets that maintained the same social status and home scar throughout all four reproductive cycles (paired: $n=56$; solitary: $n=21$). Since I predicted that egg mass production was positively associated with pair-living, this constraint was necessary to minimize the potential confounding influence of a limpet's previous social status on its egg mass production. In addition, *Siphonaria* possess a spermatheca, an organ for receiving and storing sperm (Pal et al. 2006), so limpets that change home scar locations between reproductive cycles may be able to retain sperm from previous partners. Because the data on egg mass output were non-Normal and slightly overdispersed, I used a negative binomial generalized linear model (GLM) with a log link function. The GLM was constructed using the MASS package (Venables and Ripley 2002) in R (version 3.3.1, R Core Team 2016). The dependent variable in the GLM was the total number of egg masses produced over the four cycles, and the model included social

status, shell length, and their interaction as factors. The interaction between shell length and social status was excluded from the final model because the interaction was not significant.

Tissue collection for genetic analyses

I collected samples from seven egg masses deposited by paired limpets (referred to as “paired masses”) and nine egg masses deposited by solitary limpets (“solitary masses”) for genetic parentage analysis. Because I was able to directly observe each egg mass being deposited, the maternal parent of each mass was known; for paired limpets, the partner of the maternal limpet was the “putative sire.” Five pieces were removed from each egg mass (total < 5% of the mass) using scalpel and forceps. The pieces were taken from positions haphazardly spaced around the spiral-shaped egg mass to account for potential spatial structure of paternity in the mass (i.e., uneven mixing of sperm prior to fertilization). All egg mass samples were collected during the fourth reproductive cycle, from July 17–18, so they are temporally comparable.

To sample adults, I removed each limpet from its home scar and non-destructively collected a 1 mm² piece of foot tissue with dissecting scissors. Adult tissue samples were collected from the maternal parents of 14 of 16 sampled egg masses and from the putative paternal parents of 4 of 7 paired egg masses. The remaining maternal and putative paternal limpets were not sampled because they remained clamped on their home scars and could not be removed non-destructively. In some cases, both members of a pair of limpets produced egg masses and I sampled both masses; this is true for 4 of 7 paired masses in the parentage analysis. Thus, the maternal parents for these egg masses are also considered putative sires for the egg

masses of their partners. The samples were preserved in 99% ethanol and shipped to the University of Hawai‘i at Mānoa for genetic analysis.

DNA extraction, microsatellite discovery, and development

Four *S. gigas* adults were selected for microsatellite discovery by constructing shotgun genomic libraries based on a simplified restriction-associated digestion sequencing (RADseq) protocol (after Toonen et al. 2013). First, I extracted genomic DNA from the foot tissue of each individual using the Qiagen DNeasy Blood and Tissue Kit, following the manufacturer’s protocol but collecting three separate elutions of 35, 50, and 50 µl, respectively, from the same spin column using pre-warmed 70°C water. DNA from each elution, which tend to differ in quantity and quality, was quantified using a Qubit® Fluorometer and Qubit® dsDNA HS Assay Kit (Invitrogen, ThermoFisher Scientific). For each individual, elutions were pooled starting with the first (35 µl) elution and adding the second and third elutions successively to obtain a total of at least 1 µg DNA; the pooled elutions were then concentrated by rotary evaporation. Next, DNA from the four limpets was digested in separate reactions containing 272–471 ng DNA, 1 µl DpnII, 5 µl NEBuffer™ 3.1 (New England Biolabs), and water to a total volume of 50 µl. The samples were incubated at 37°C for 3 hours followed by 20 minutes at 65°C, then purified with AMPure XP beads (Beckman Coulter).

Four genomic libraries were prepared with the digested DNA from each of the four individuals using the KAPA Hyper Prep Kit (Kapa Biosystems Ltd.) and Illumina TruSeq adapters (Illumina Inc.). The libraries were sequenced on an Illumina MiSeq platform with V3 chemistry and 600 cycles to produce 300 bp paired-end reads; all sequencing was carried out at

the University of Hawai‘i Advanced Studies in Genomics, Proteomics and Bioinformatics. The resulting sequences from each library were trimmed and assembled with SeqMan NGen 12 (DNASTAR, Inc.) with a minimum quality of 30, minimum length of 50 bp, and minimum depth of ten reads per contig. The assembled sequences were then imported to Geneious version 11.1.4 (<http://www.geneious.com/>) and trimmed a second time using BBDuk (Bushnell, BBDuk Geneious plugin) to remove adapters, with the same minimum quality and length parameters.

To isolate microsatellites in each of the four assembled libraries, I utilized Phobos Tandem Repeat Finder (Mayer, Phobos 3.3.11 Geneious plugin). I searched for perfect di-, tri- and tetra-nucleotide motifs with a minimum of six repeat units. The microsatellites were manually screened and a subset of 22 was selected for further development based on: number of repeat units, depth of coverage, absence of other repetitive sequences flanking the microsatellite, presence in more than one library, evidence of allelic variation, and ability to design primers flanking the microsatellite. I used Primer3 version 2.3.7 (Untergasser et al. 2012, Primer3 Geneious plugin) to design locus-specific primers with optimal melting temperature of 60°C, optimal length at 20 bp, and product size from 100–300 bp.

I extracted DNA from adult tissue (from maternal and putative paternal limpets) using the Qiagen DNeasy Blood and Tissue Kit, following the manufacturer’s protocol but with two separate elutions in 30 µl buffer EB. To obtain embryo DNA, samples from five different locations on the same egg mass were pooled in one tube. Mean embryo density, determined by counting the number of embryos in samples from 17 egg masses, was 47 embryos/mm³ (SD=24). Thus, each 2x1x1 mm piece of egg mass contained approximately 100 embryos, resulting in pooled samples of approximately 500 embryos for each egg mass. I used a Qiagen kit to extract

DNA from the pooled embryo samples but with the following modifications: first, to break up the egg masses, 540 μ l buffer ATL and 0.1 g 0.1 mm zirconia/silica beads were added to each tube and the samples were agitated by shaking for 5 minutes at 25 Hz, then incubated for 10 minutes at 56°C. The agitation and incubation steps were repeated, followed by addition of 60 μ l proteinase K and incubation at 56°C for approximately 12 hours. The rest of the extraction followed the Qiagen kit protocol as written but with two separate elutions of 30 μ l in buffer EB. This yielded final concentrations of embryo and adult DNA ranging from 1–15 ng/ μ l.

PCR and amplicon sequencing

Microsatellites were amplified using a two-step amplification protocol: the first polymerase chain reaction (PCR) step utilized locus-specific primers to amplify the locus of interest, and a second PCR step utilized barcode primers to tag individual samples with combinatorial barcodes, following Vartia et al. (2016). A 5' adapter sequence was added to each locus-specific primer to facilitate binding of barcode primers in the second PCR step. The barcode primers consisted of the complement to an adapter sequence and one of twelve barcode sequences. Forward primers were adapted with Hill, M13, or Neo and reverse primers were adapted with CAG (sequences provided in Vartia et al. 2016). I tested the locus-specific primers with 5' extensions for secondary structure formation using the IDT Oligo Analyzer tool (<http://eu.idtdna.com/calc/analyzer>) and ThermoFisher Multiple Primer Analyzer (<https://www.thermofisher.com>), and I designed primers to minimize formation of hairpins, primer dimers, and hetero-dimers. Four forward and eight reverse 10 bp barcode sequences were utilized for a total of 32 unique combinatorial barcodes, which enabled me to pool samples prior

to sequencing and demultiplex the sequences based on their forward-reverse barcode combination. Some combinatorial barcodes assigned to adult limpets were repeated in the embryo samples; however, amplicons from adults and embryos were always pooled in separate libraries for sequencing, so the samples could be distinguished even though they shared the same barcode sequences.

Microsatellites were amplified individually or in multiplex PCRs containing 2–4 primer pairs. Each pair of locus-specific primers were first tested individually to optimize annealing temperature and number of cycles, and multiplexes were formed by combining primers that amplified under similar cycle conditions. Each 10 μ l multiplex reaction consisted of 0.5 μ l template DNA, 0.2 μ mol each forward and reverse primer, and 5 μ l MyTaq Red Mix (Bioline). In addition, 0.5 μ l DMSO and 0.5 μ l BSA were added to PCR reactions for embryo samples (except for locus MS-26, which did not include the additives and utilized 0.5 μ l of 1:10 diluted embryo DNA as the template). Annealing temperatures (T_a) for the multiplex PCRs ranged from 55–60°C depending on the primers; thermocycling profiles consisted of a 90 s initial denaturation step at 94°C, followed by 25–30 cycles of 20 s at 94°C, 20 s at T_a , 25 s at 72°C, and a final extension for 10 minutes at 72°C.

After the first PCR, products were individually purified either by bead cleaning or with the Qiagen PCR purification kit. Bead cleans utilized a 1:1.8 ratio of PCR product to AMPure XP reagent to wash out fragments < 100 bp. The two purification methods produced similar results, and the cleaned amplicon DNA was used as template for the subsequent barcode PCR. To incorporate barcodes, 10 μ l PCRs were carried out containing 2 μ l template, 0.2 μ mol of each forward and reverse barcode primer per microsatellite locus, and 5 μ l MyTaq Red Mix. The

thermocycling profile for all barcode PCRs consisted of 90 s of initial denaturation at 94°C, 8 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, and a final extension for 5 minutes at 72°C.

Products from the barcode PCR were purified either by gel purification or bead cleaning with AMPure XP reagent. For the gel purification method, the PCR products were run on 1.3% agarose and bands corresponding to expected product size were cut out, then amplicon DNA was isolated using the Qiagen gel purification kit. The cleaned products were quantified using a Qubit® Fluorometer and Qubit® dsDNA HS Assay Kit; both purification methods produced similar results and yielded 20–340 ng DNA. The samples, including negative controls, were pooled into libraries containing approximately equal amounts of amplicons from each individual and locus.

The libraries were prepared for sequencing and Illumina TruSeq adapters were incorporated using the KAPA Hyper Prep Kit. Amplicon libraries were sequenced on an Illumina MiSeq platform with V3 chemistry and 600 cycles to obtain at least 2,000 reads per locus per individual. Raw reads from the amplicon libraries were trimmed using BBduk in Geneious with a minimum quality of 30. This step also removed Illumina adapters and discarded reads < 50 bp, as the expected microsatellite-containing amplicons were > 100 bp. The trimmed reads were paired by name and merged, then sequences in each library were separated by barcode in Geneious and a fastq file was created for each individual (adult or egg mass).

Microsatellite genotyping and parentage analysis

I utilized the program MEGASAT 1.0 (Zhan et al. 2017) to demultiplex and process amplicon sequence data from adult limpets and egg masses. MEGASAT was designed to score

microsatellite genotypes from multiplexed next generation sequence data. The software applies a series of decision rules to distinguish true microsatellite alleles from PCR artefacts (e.g., amplification stutter) and account for amplification bias among alleles (Zhan et al. 2017). The inputs for MEGASAT are a primer file and a set of fastq files. In this case, each fastq file contained sequences from one adult limpet or one egg mass. The primer file included information used to identify reads containing microsatellite regions of interest: the sequences of locus-specific microsatellite primers, 5' and 3' flanking sequences for each locus, and the microsatellite repeat unit. The user can also specify "ratios group" values, which control the stringency of MEGASAT in identifying microsatellite alleles compared to PCR artefacts (Zhan et al. 2017). I used the default ratios group values in MEGASAT.

For adult limpets only, MEGASAT was used to assign genotypes to each individual at the 21 microsatellite loci sequenced. I then utilized CERVUS version 3.0.7 (Tristan Marshall, Field Genetics Ltd., Kalinowski et al. 2007) to calculate the expected heterozygosity, null allele frequencies, and exclusion probabilities for each locus. Loci were tested for deviations from Hardy Weinberg equilibrium (HWE) using a chi square goodness of fit test in CERVUS with Bonferroni and Yates corrections and a minimum expected allele frequency of one.

Histograms of read length for each individual and locus were generated using MEGASAT. I manually checked the genotype of each limpet by comparing the pattern of peaks in its read length histogram to the MEGASAT-assigned genotype for that limpet. A diploid individual cannot have more than two alleles per locus; however, the read length histograms for some loci contained numerous peaks of similar size attributed to stutter, making it difficult to determine which read lengths corresponded to true alleles and which were PCR artefacts. Loci

with complex peak patterns in the read length histograms were excluded from the parentage analysis due to ambiguity in scoring genotypes. Out of 21 microsatellite loci sequenced, four were chosen for use in the parentage analysis because they met all of the following conditions: polyallelic, consistent with Hardy Weinberg equilibrium, and unambiguous to score.

Focusing on these four loci, I used MEGASAT to assign genotypes and generate read length histograms for the egg mass samples. MEGASAT will call a maximum of two alleles per locus per sample. However, each egg mass sample contained DNA from a pool of approximately 500 embryos, so their collective genotype may consist of four alleles per locus (two maternal and two paternal), or more than four alleles if multiple sires fertilized offspring in the same egg mass. To account for this, I examined the read length histograms of egg mass samples and manually corrected MEGASAT-assigned genotypes when there were more than two alleles present. A conservative approach was taken and egg masses were only scored for alleles that were present in at least one adult in the study.

I compared the genotypes of each egg mass to its known maternal parent and, for paired masses, the putative paternal parent. A minimum number of sires was calculated for each egg mass at each locus by dividing the number of non-maternal alleles present in the mass by two (i.e., assuming all sires were heterozygous) and rounding up to the nearest integer. I also took a less conservative approach, assuming all sires were homozygous, and estimated the number of sires as equal to the number of non-maternal alleles present in the egg mass.

Paired egg masses were categorized as either consistent with genetic monogamy (if all alleles present in the egg mass were found in the maternal and/or putative paternal parent) or not

consistent with monogamy (egg mass contained at least one allele not found in the maternal and putative paternal parent). The latter case provides evidence for extra-pair mating.

Probability of detecting multiple paternity

I used a model developed by Neff and Pitcher (2002) to assess the power of my panel of markers to detect multiple paternity in *S. gigas* egg masses. The probability of detecting multiple mating (PrDM) was calculated for masses sired by two or four limpets and with a range of paternal skew scenarios, assuming in each case that 100 embryos (the maximum allowed by the software) were sampled and genotyped.

Results

A total of 37 solitary and 74 paired *Siphonaria gigas* (i.e., 37 pairs) were marked with individual ID numbers at the beginning of the study. The distance between solitary limpets and their nearest neighbor ranged from 2.5–140.5 cm (median 25.3 cm). The two social classes were similar in shell length (Wilcoxon rank-sum test, $z=-1.76$ $p = 0.0777$), with mean shell length of 50.6 (SD=6.9) mm in paired and 47.2 (SD=6.0) mm in solitary limpets (Fig. 2). Three solitary and two paired limpets were lost before the end of the study period, so data on their behavior was gathered up to the point where they disappeared.

Behavior and social status

Almost one third (34 of 111) of initially paired and solitary limpets changed social status over ten weeks of observation. The remaining limpets (21 of 37 solitary and 56 of 74 paired) maintained their original social status and home scar location. Almost half (15 of 37) of solitary limpets became paired by the end of the study: 12 joined another solitary or unmarked limpet to form a new pair, while three solitary limpets replaced one member each from three existing pairs (Fig. 3). Four solitary limpets became part of a group of three or more limpets.

Paired limpets tended to remain in pairs. Only 2 of 37 pairs underwent separations where both became solitary. In one case the leaving partner established a new home scar 250 cm away from its former partner (which remained in the old location), and in the second case the leaving limpet moved 30 cm away from its former partner. Two paired limpets from separate pairs became solitary when their partners left and established home scars near different limpets. Three additional paired individuals became solitary and were each replaced by another limpet; two of

these newly solitary limpets were found inhabiting the old scar of the solitary individual that replaced them (Fig. 3). Five pairs of limpets became trios when a third limpet established a new home scar adjacent to the paired limpets' existing scars.

I observed *S. gigas* individuals mating both with their social partner (within-pair) and with limpets that were not their social partner (extra-pair). Over 10 weeks, 11 pairs of limpets were observed mating within-pair, and 3 of 11 were observed mating twice within-pair. In the three pairs that mated twice within-pair, the second mating event occurred 3, 13, and 18 days after the first mating event, respectively. I also observed seven extra-pair mating events, including four instances where two limpets from different pairs mated with each other. Solitary limpets were observed mating with other solitary limpets (n=2), a paired limpet (n=1), and unmarked limpets (n=5).

Egg mass production

Egg mass production varied between individuals, across reproduction cycles, and by social status. On a single reproductive cycle the minimum number of egg masses produced per individual was zero and the maximum was two. Over four reproductive cycles, total egg mass production ranged from zero to five masses per individual.

Egg mass output was related to limpet shell length (Fig. 4) as well as social status (Fig. 5). When tested with a generalized linear model, the covariate shell length was significant ($z=3.10$, $p=0.0019$), although it explained only 14.2% of variation in egg mass production. The relationship between social status and egg mass production was marginally nonsignificant at $\alpha=0.05$ ($z=-1.77$, $p=0.0762$). However, paired limpets produced almost twice as many egg

masses as solitary limpets: the mean total egg mass output over four cycles was 1.57 (SD=1.37) and 0.81 (SD=1.08) for paired and solitary limpets, respectively (Fig. 5).

Microsatellite discovery and genetic analysis

Sequencing of the four genomics libraries yielded from 204,558 to 1,683,261 reads (a total of 3,384,326 reads). Out of the 22 candidate microsatellite loci identified from these libraries, PCR was successful for 21 loci and one locus failed to amplify.

Adult limpets (n=19) and their egg masses (n=16) were genotyped at four polymorphic microsatellite loci (Table 1). Each locus contained 4–7 alleles of different length, and sequence data provided information on the cause of allelic variation at these loci. Nearly all length variation was due to differences in the number of microsatellite repeat units. Along with number of repeat units, locus MS-34 contained a poly-A region within the flanking region that contributed to allele length variation at this locus. Two alleles contained nucleotide substitutions, but because each substitution was always associated with a certain unique number of repeats, these nucleotide polymorphisms did not create any hidden allelic variation.

Allele frequencies in the adults confirmed that the four loci were suitable for parentage analysis. The loci did not deviate significantly from Hardy-Weinberg equilibrium ($p > 0.05$) and none showed evidence of null alleles, with estimated null allele frequencies < 0.04 . The mean number of alleles per locus was 5.75 and expected heterozygosity was 0.691. The mean expected homozygosity was 0.307 across the four loci, and the mean probability of two individuals being homozygous at one locus was $0.307^2 = 0.0942$. The combined probability of exclusion for this set of four loci, that is, the probability of excluding a random unrelated individual as a parent for

a given offspring when neither parent is known based on their multilocus genotypes, was 0.764. With one parent known, the combined probability of exclusion for a second unrelated individual was 0.926.

Genetic analysis of egg mass samples identified between 1–5 alleles per locus per mass. The egg masses contained at least one maternal allele at every locus genotyped. Genotype data could not be obtained for one locus each for three egg masses due to low read depth, so parentage analysis of those masses was based on just three loci.

Parentage analysis

Three out of sixteen egg masses showed evidence of multiple paternity with a minimum of two sires (Table 3). For all three cases of multiple paternity, the maternal limpet was solitary. The remaining 13 of 16 egg masses were consistent with single paternity, since none contained more than two non-maternal alleles at a given locus. Applying the less conservative approach of assuming all sires were homozygous, the number of egg masses with evidence of multiple paternity at one or more loci increased from three to eight egg masses, and the number of sires per egg mass ranged from 1–4 instead of 1–2.

I found evidence of extra-pair paternity in three out of four paired egg masses for which both the maternal and putative paternal limpet were sampled (Table 2). Only one egg mass was consistent with sexual monogamy, meaning that all identified offspring alleles in that mass were found in either the maternal limpet or its social partner. The other three egg masses contained alleles at two or more loci that were not present in either the maternal or putative paternal limpet, indicating extra-pair paternity. All three of these egg masses contained 1–2 non-maternal alleles

at each locus including at least one allele not present in the putative father; thus, they were compatible with a single extra-pair sire (assuming sires were heterozygous, the most conservative approach). Two out of three of the masses with extra-pair paternity lacked alleles from the social partner at one or more loci, excluding this individual as a sire. The alleles present in the third egg mass were compatible with either one extra-pair sire or with two sires: the social partner of the maternal limpet and one extra-pair sire.

Probability of detecting multiple paternity

The power to detect multiple paternity with this set of four loci was high for a range of paternity scenarios (Table 4). With at least 100 offspring genotyped, the probability of detecting multiple paternity (PrDM) was 94% even when paternity was highly skewed between two males siring 95 and 5% of offspring, respectively. PrDM decreased only when the second sire's contribution was very small (Table 4).

Discussion

Genetic data revealed that *Siphonaria gigas* can be polygamous and produce egg masses sired by more than one individual. That said, multiple paternity was detected in only 19% (3 of 16) of egg masses, a relatively low frequency compared to other marine gastropods (Angeloni et al. 2002, Brante et al. 2011, Dupont et al. 2006, Morales et al. 2016, Walker et al. 2016, Xue et al. 2014). For example, multiple paternity was found in 89.5% of broods in a muricid (Xue et al. 2014), and Kamel and Grosberg (2012) detected multiple paternity in 100% of *Solenosteira* broods. The number of sires contributing to a single egg clutch (i.e., the degree of multiple paternity) ranged from 2–8 in marine gastropods (Morales et al. 2016, Walker et al. 2007, Xue et al. 2014). In contrast, there was no genetic evidence of more than two sires per egg mass in *S. gigas* when the same approach of estimating minimum number of sires was applied. When a less conservative method was used to calculate the number of sires, assuming all sires were homozygous, the frequency of multiple paternity in *S. gigas* increased from 19% to 50% (8 of 16 masses) and the number of sires ranged from 1–4. However, the low probability of two individuals being homozygous at one locus (0.0942) makes the latter estimates less realistic, especially considering information from four loci was used to determine the number of sires. In either case, compared to other marine gastropods with more promiscuous mating systems, the number of sires contributing to *S. gigas* egg masses at Punta Culebra was relatively low.

While the majority of *S. gigas* egg masses were compatible with single paternity, that conclusion depends on the power of the genetic markers to detect more than one sire. Considering several hundred embryos were sampled and pooled from each egg mass, our panel of microsatellites provided a high probability of detecting multiple paternity (PrDM) of ≥ 0.94

even assuming paternity was highly skewed at 95:5 between two males. This level of paternity skew would be more extreme than typical levels reported in marine gastropods such as knobbed whelks (Walker et al. 2007) and slipper shells (up to five males siring 10.7–46.3% offspring each, Brante et al. 2011). Thus, the PrDM of my analysis was sufficient to detect multiple paternity in biologically realistic scenarios, strengthening the finding that multiple mating occurs at a relatively low frequency in this population of *S. gigas*.

Multiple mating is common among hermaphroditic gastropods (Nakadera & Koene 2013) and among pulmonates (Jordaens et al. 2007), appearing to be the trend rather than the exception. The discovery of multiple paternity in *S. gigas* conforms to these patterns, yet the low frequency and degree of multiple paternity distinguishes *S. gigas* from closely related taxa. Proposed benefits of multiple mating include enhanced genetic diversity of offspring, facilitation of female choice, and (for males) insurance against predation of inseminated females (Arnqvist and Nilsson 2000). On the other hand, sperm competition among males increases with group size and the rate of multiple mating (Charnov 1980, Tan et al. 2004). The mating system of *S. gigas* is likely influenced by most if not all of these factors, and the most adaptive reproductive strategy must balance male and female fitness.

Genetic results also confirmed that social partners in *S. gigas* are not sexually monogamous: three out of four egg masses, for which both the maternal genotype and that of the putative sire were known, displayed evidence of extra-pair paternity at two or more loci. Although the sample size was small, the frequency of extra-pair paternity (75%) was supported by direct observations of extra-pair mating.

Paired limpets in the field mated both within-pair and extra-pair, albeit more often within-pair (fourteen versus seven times). These results are consistent with previous work on *S. gigas* (Levings and Garrity 1986), in which 69% of mating events occurred between limpets who were nearest neighbors (although Levings and Garrity did not differentiate between solitary and paired limpets). Tracking the movements and mating behavior of marked paired and solitary individuals demonstrated that limpets living in pairs also mated with limpets who were not their social partner (i.e., limpets who were not their nearest neighbor).

I found that patterns of mating can be asymmetric between two members of a social pair. For example, one pair of limpets was observed mating with each other twice—once 13 days before depositing their egg masses and again five days after. Parentage analysis of the egg masses produced by each pair member revealed that one limpet's egg mass was sired within-pair, while the other's mass contained alleles from an extra-pair sire, indicating the second pair member had mated with another limpet in addition to its social partner. Thus, one limpet received sperm from its partner, while the other either did not receive or did not utilize sperm from its partner to fertilize its eggs. These observations suggest that copulation is not reciprocal in *S. gigas*, even between social partners, consistent with the finding of non-reciprocal copulation in *Siphonaria capensis* (Pal et al. 2006).

Social monogamy does not entail sexual monogamy in other taxa, suggesting that pair-living may be beneficial despite ubiquitous extra-pair mating (Fietz et al. 2000, Griffith et al. 2002, Ophir et al. 2008). As in these taxa, evolutionary drivers of pair-living in *S. gigas* must not rely on exclusive production of offspring between social partners. The finding of multiple mating in solitary limpets, along with the behavioral and genetic evidence of extra-pair mating in paired

limpets, seemingly contradicts the notion that pairing is a strategy to alleviate limited mate access. However, pair-living may still be beneficial if encounters with potential mates are sporadic and limited. *S. gigas* may mate opportunistically with individuals they encounter while foraging off scar. However, since activity is restricted and the majority of time is spent on scar, establishing a home scar near a partner could ensure that limpets always have access to a mate, including times when they fail to encounter other limpets while foraging.

Over four reproductive cycles, I found that paired limpets produced more egg masses than solitary limpets, consistent with the idea that one benefit to pair-living is greater female reproductive success. While this relationship was marginally nonsignificant, the difference was substantial, with nearly two-fold greater egg mass output in paired limpets. It is possible that solitary limpets produce fewer, but larger egg masses containing more embryos, or that offspring survival differs by social status. However, the proportion of viable embryos in paired and solitary egg masses did not differ after eight days of development (J. Schaefer, unpublished data), which is when embryos begin to hatch from egg masses and enter a planktonic phase.

The observation that paired *S. gigas* tended to produce more egg masses than solitary *S. gigas* is consistent with the environmental constraints hypothesis for social monogamy (Baeza and Thiel 2007). Previous studies have established a link between a symbiotic lifestyle, constraints on mate access, and social monogamy in other tropical marine invertebrates; in these organisms, a high risk of predation outside of the host is thought to limit movement and favor social monogamy (Baeza 2008, Baeza 2010, Pfaller et al. 2014). Although *S. gigas* is not symbiotic, individuals occupy specific home scars in the intertidal that provide refuge from harsh ecological conditions, similar to the function of the hosts of symbiotic marine invertebrates.

Since *S. gigas* cannot self-fertilize, they must receive sperm from other individuals to produce fertilized egg masses. Therefore, given their limited periods of movement and the importance of homing in *S. gigas* (Garrity 1984, Garrity and Levings 1983), pairing may be beneficial if establishing a home scar directly adjacent to a partner facilitates mate access.

Status switches from solitary to paired occurred more often than paired to solitary transitions, further suggesting a preference for pair-living during this time period. Over the 10-week study, 40% (15 of 37) of solitary limpets became paired and only 12% (9 of 74) of paired limpets became solitary; those that did become solitary were often replaced by a different limpet. In these cases, I could not determine whether the incoming limpet inhabited the home scar of the replaced limpet first, preventing the replaced limpet from returning to its scar, or whether the replaced limpet intentionally moved away to establish a new scar, leaving its old scar vacant. The first scenario would indicate there is competition for partners, while both scenarios suggest there may be benefits to occupying the previous home scar of another limpet.

Seasonal changes in abiotic factors have potential to drive social organization. The social dynamics observed at Punta Culebra suggest that the relative benefits of living in pairs vs. being solitary could vary seasonally or over the lifetime of *S. gigas* individuals. For example, *S. gigas* behavior and activity patterns may be different in the rainy season (May–December) than in the dry season (January–April), since *S. gigas* typically move only when the substrate is wet at low tide (Garrity 1984). Additionally, a reduction in upwelling-driven nutrient availability in the Gulf of Panama during the rainy season (D’Croz and O’Dea 2007) could lead to lower algal biomass and place energetic constraints on *S. gigas* movement. Lombardo et al. (2013) showed that 75% of limpets at Punta Culebra were paired in June–July 2004, while I observed a trend of solitary

individuals moving into pairs from May–June 2016. Together, these studies point to the value of living in pairs during the early- to mid-rainy season.

If mate access is a key driver of pair-living, the question remains as to why *S. gigas* are not more commonly found in larger groups. One possibility is that competition becomes more intense when more than two limpets form home scars and graze in close proximity. Given their large body size and the low biomass of cyanobacterial crust on which they feed, it seems likely that *S. gigas* are resource limited. This idea could be tested by examining variation in group size within or among populations with respect to habitat quality/algal abundance. Furthermore, seasonal upwelling and variation in productivity could moderate resource availability, influencing the relative value of different social strategies in *S. gigas*. Long-term tracking of marked individuals would shed light on temporal patterns in social organization with respect to seasonal abiotic factors.

Conclusion

Behavioral observations of mating combined with genetic parentage analysis indicated that *S. gigas* are not sexually monogamous, including those that live in pairs. Extra-pair paternity and multiple paternity occurred within a single reproductive cycle. The trend toward greater egg mass output of paired limpets, combined with the fact that the majority of limpets at Punta Culebra live in pairs, suggests that pairing confers reproductive benefits. Hermaphroditic animals gain fitness through both male and female reproductive functions, and the influence of social status on male reproductive success of *S. gigas* is unknown. To test for adaptive benefits of pair-living, future studies should measure total fitness, including both male and female reproduction

and in terms of the number of offspring produced. Future work should also incorporate genetic sampling of offspring over multiple reproductive cycles to examine temporal patterns in mating and parentage. In addition, studies manipulating the social status of individuals in the field and then monitoring their behavior and reproduction could further elucidate the fitness consequences of pairing in this hermaphroditic gastropod.

Tables

Table 1. Summary statistics for microsatellite loci used in the parentage analysis. Locus name, repeat unit, number of alleles (N_a), number of adults genotyped at that locus (N), observed heterozygosity (H_o), expected heterozygosity (H_e), probability of deviation from Hardy-Weinberg equilibrium (HW), estimated null allele frequency (F_{null}), and exclusion probability (P_{excl}). The exclusion probability is the probability of excluding a random unrelated individual as parent for a given offspring based on their genotypes at that locus. Hardy Weinberg equilibrium was tested using a chi square goodness of fit test in CERVUS with a minimum expected allele frequency of one.

Locus	Repeat	N_a	N	H_o	H_e	HW	F_{null}	P_{excl}
MS 03	AC	5	19	0.684	0.724	>0.05	0.0222	0.292
MS 26	TAA	7	19	0.895	0.856	>0.05	0.033	0.494
MS 31	TC	7	18	0.556	0.603	>0.05	0.0269	0.202
MS 34	TTG	4	19	0.526	0.587	>0.05	0.0276	0.175

Table 2. Results from the test for monogamy in paired egg masses: alleles present in the pooled offspring from egg masses (O) and genotypes of their respective maternal (M) and putative paternal (PP) limpets. The putative paternal limpet was the social partner of the maternal limpet. Parentage was classified as extra-pair (EP) or consistent with sexual monogamy (MO). Minimum number of sires was calculated by dividing the number of non-maternal alleles present at a single locus in the egg mass by two and rounding up to the nearest integer. Bold font indicates alleles present in the egg mass that were not found in either the maternal or putative paternal limpet for that mass, indicating extra-pair paternity.

Egg mass	MS-03			MS-26			MS-31			MS-34			Parentage
	O	M	PP	O	M	PP	O	M	PP	O	M	PP	
20	113 117 119	113 119	115 115	283 287 295	287 295	272 277	57 59 61	57 57	57 57	235 238 244	235 238	238 238	EP, min 1 sire
27	115 117	115 117	117 117	275 277 281	277 281	275 275	43 57 61	57 61	43 57	235 238	235 238	238 238	MO, min 1 sire
30	115 117	117 117	115 117	275 281	275 275	277 281	57 59	43 57	57 61	232 238	238 238	235 238	EP, min 1 sire
33	115 117	115 115	115 117	275 277 287 295	277 295	281 283	57 57 57	57 57	57	235 238	238 238	232 238	EP, min 1 sire

Table 3. Results from the test for multiple paternity in paired and solitary egg masses: alleles present in the pooled offspring from egg masses (O), genotype of the maternal limpet (M), and social status of the maternal limpet: paired (P) or solitary (S). The maternal genotype is known for these masses, except for two masses for which the maternal parent was not sampled (NS). No data (ND) indicates an individual could not be genotyped at that particular locus. Bold font indicates alleles present in the egg mass that were not found in the maternal limpet for that mass. The minimum number of sires was determined by counting the number of non-maternal alleles present in the offspring, dividing by two, and rounding up to the nearest integer.

Egg mass	Maternal social status	MS-03		MS-26		MS-31		MS-34		Min # sires
		O	M	O	M	O	M	O	M	
21	P	115 117 121	115 121	275 281 295	275 281	57 59	57 59	238 238	238	1
23	P	115 117 121	NS	275 281 295	NS	57 59	NS	238 NS	NS	1
25	S	115 117 121	117 121	272 277 281 283	272 283	57 61	57 57	235 238	235 238	1
26	S	115 119	115 119	275 287	281 287	53 57 59 61 63	57 57	234 238 244	232 238	2
28	S	113 115 119	115 117	275 277 283 287	275 277	57 61 63	57 57	232 238	238 238	1
29	S	115	113 115	ND	275 287	57	57 57	232 235 238	235 238	1

31	P	117	117 117	275 287	283 287	57 59	59 61	235 238 244	238 244	1
32	S	115 117 119	117 117	269 275 283 287	287 295	ND	51 51	232 235 238 244	238 238	2
34	S	113 115	115 115	277 281 283	281 283	41 53 57	41 57	232 235 238	235 238	1
35	S	115 117 121	113 115	275 281 287	275 287	57 59	57 59	235 238	235 238	1
36	S	115 117	NS	277 281 283	NS	57 59	NS	232 238	NS	1
37	S	ND	117 119	275 277 281 283	277 283	57 59	51 57	232 235 238 244	238 238	2

Table 4. Probability of detecting multiple paternity (PrDM) for different numbers of sires and levels of paternity skew (e.g., skew of 50:50 indicates two males each sired 50% of offspring in an egg mass). Each simulation assumes 100 offspring were genotyped.

Number of sires	Paternal skew (% offspring sired)	PrDM
2	50:50	0.970
2	80:20	0.970
2	95:5	0.940
2	99:1	0.527
4	25:25:25:25	> 0.999
4	90:3.3:3.3:3.3	0.999

Figures

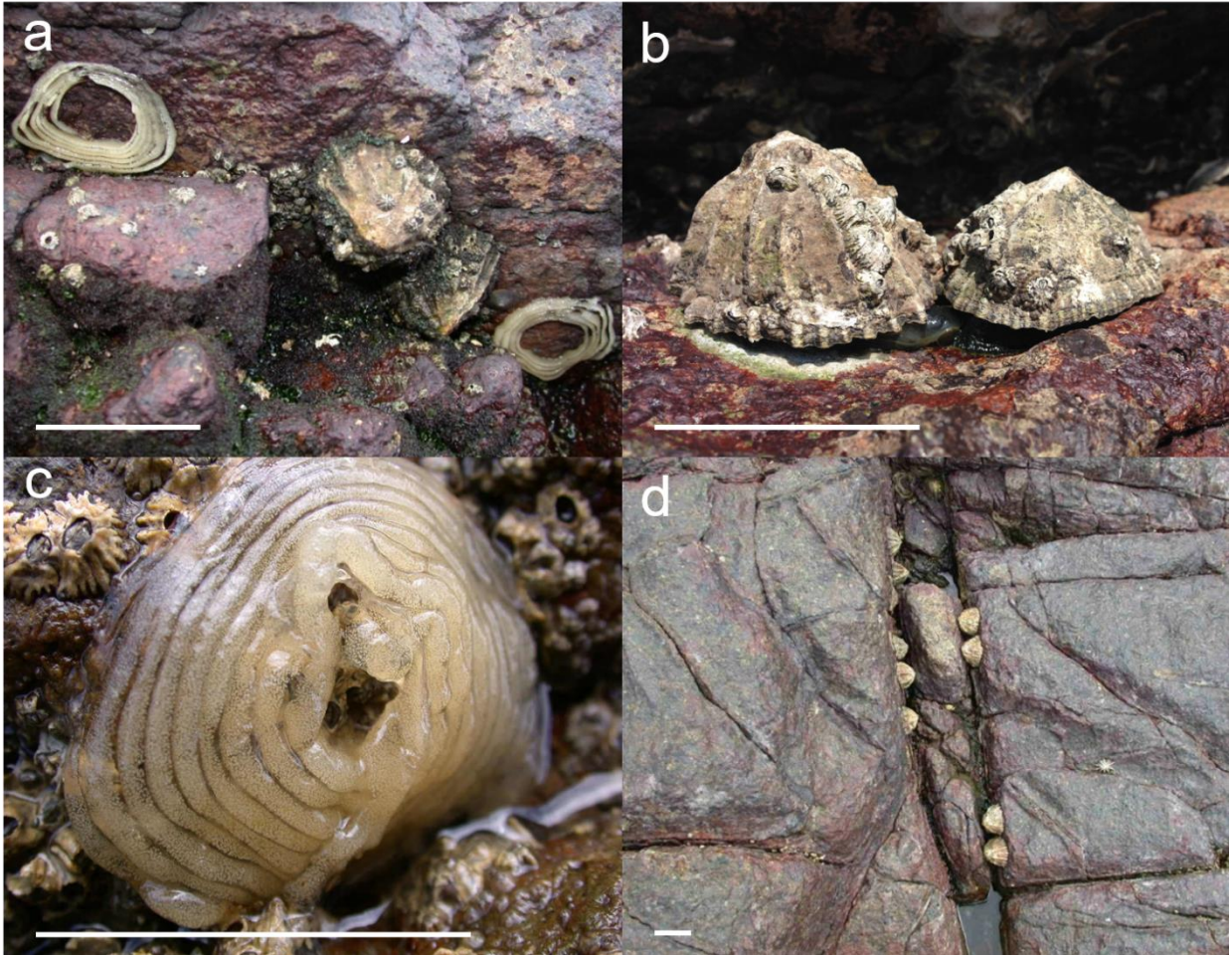


Figure 1. *S. gigas* and their egg masses at Punta Culebra: (a) pair of limpets on home scars and their egg masses; (b) limpets mating; (c) *S. gigas* egg mass; (d) paired and solitary limpets on their home scars at Punta Culebra. White bars indicate approximately 5 cm. Photographs by J. H. Christy; (d) was previously published in Lombardo et al. (2013).

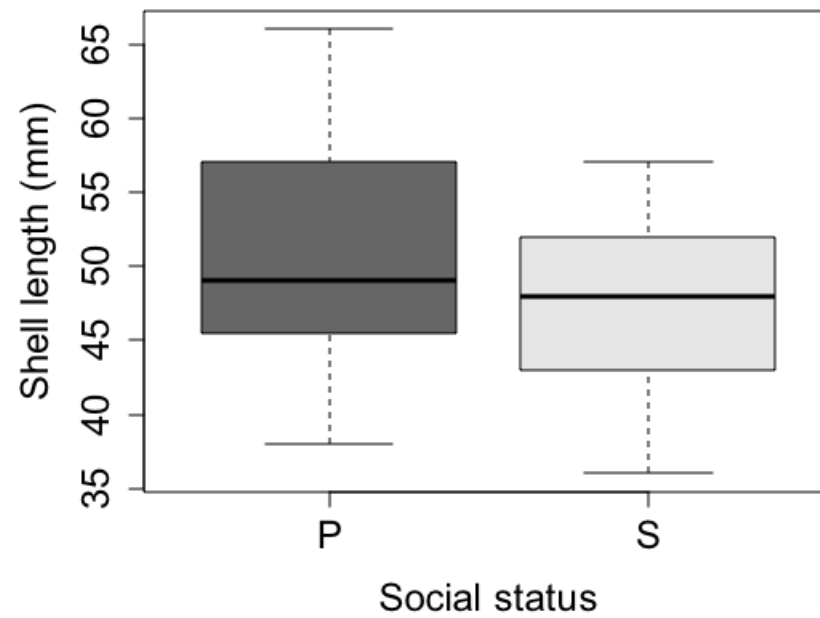


Figure 2. Shell length of paired (n=21) and solitary (n=56) limpets from which behavior and egg mass data were collected.

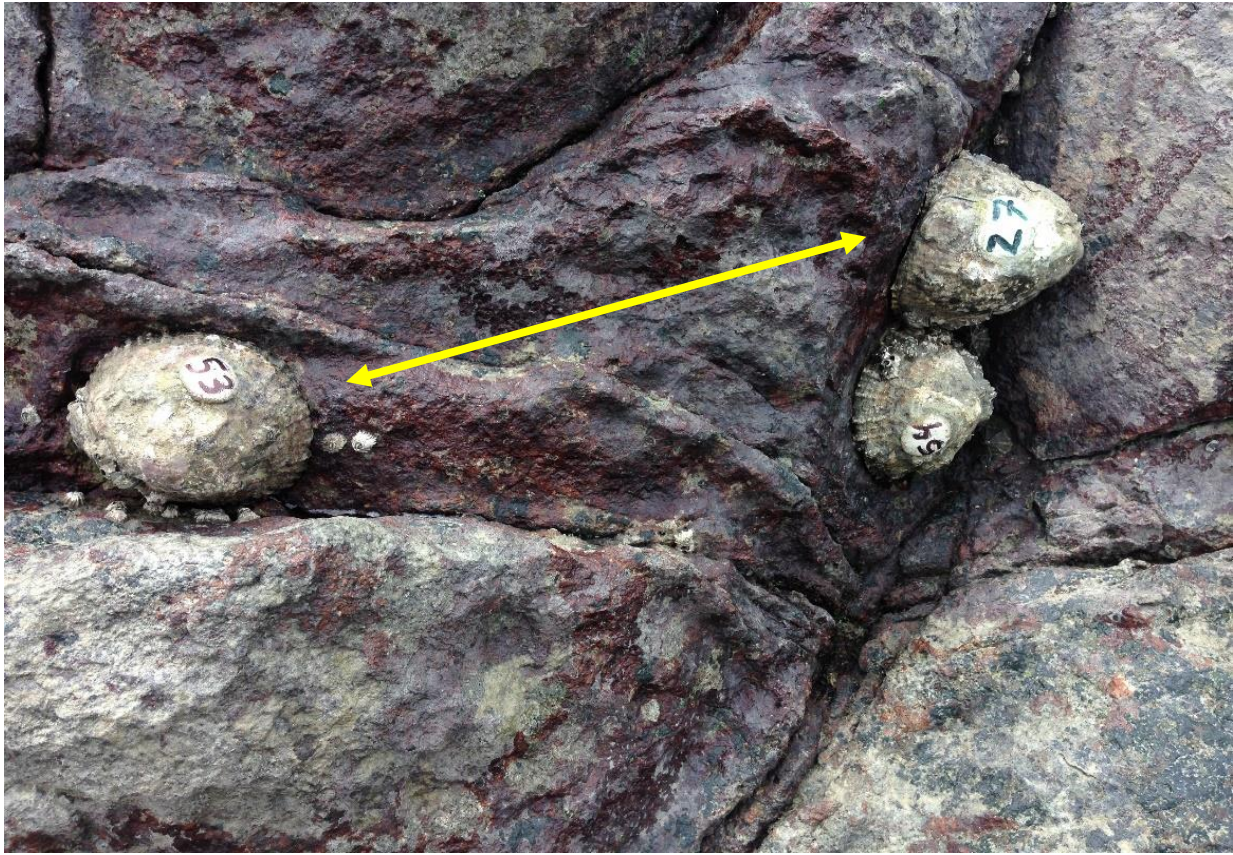


Figure 3. Social status change involving a pair and nearby solitary limpet. The limpets marked 53 and 54 were initially paired, and limpet 27 was initially solitary. This photo shows all limpets sitting on their new home scars after the status change (indicated by the arrow), where limpets 27 and 53 switched locations. Photograph by J. Schaefer.

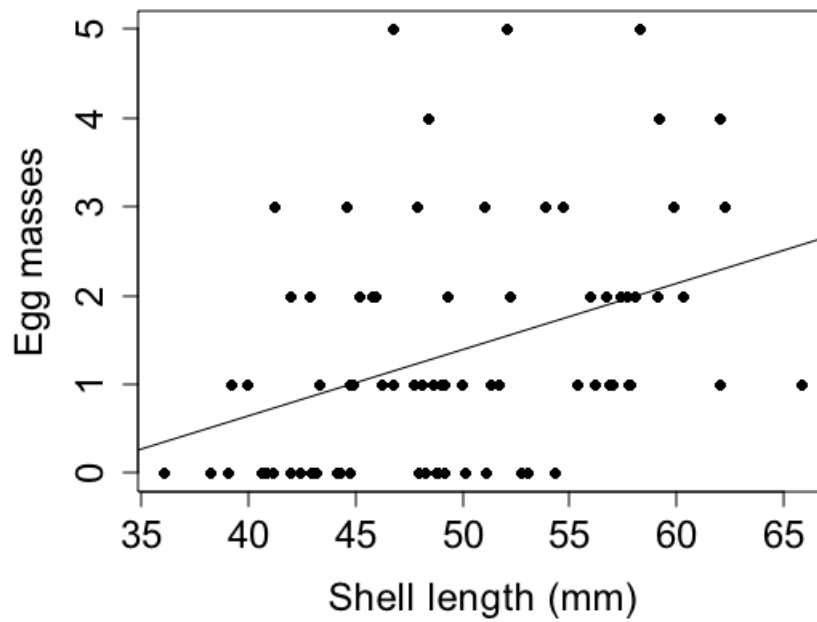


Figure 4. Total egg mass production over four reproductive cycles plotted against shell length of paired and solitary limpets. There was a significant, but weak positive association between length and the number of egg masses produced (GLM, $z=3.10$, $p=0.0019$). Points are jittered for clarity.

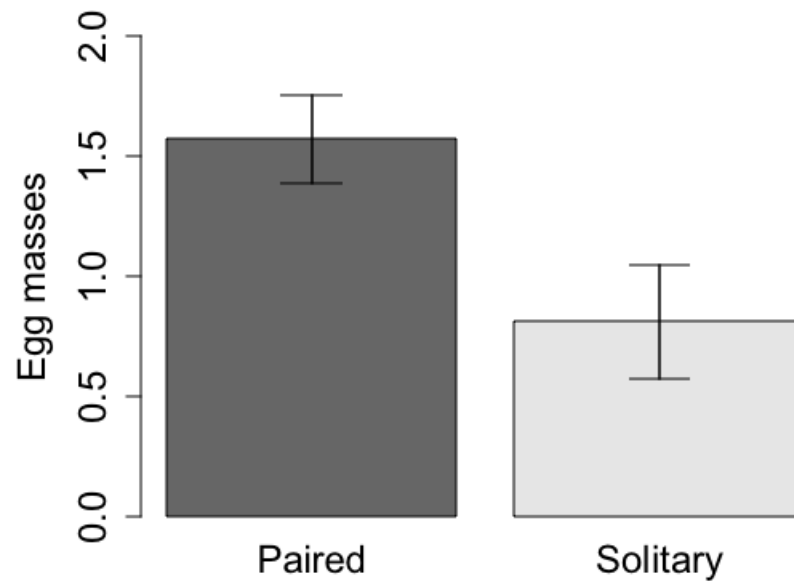


Figure 5. Mean number of egg masses produced by paired (n=56) and solitary (n=21) limpets over four reproductive cycles. Error bars indicate standard error.

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